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**(71) Applicant:** FUJIREBIO KABUSHIKI KAISHA also trading  
as FUJIREBIO INC.  
6-7, Shimoochiai 4-chome Shinjuku-ku  
Tokyo 161(JP)

**(72) Inventor:** Ashihara, Yoshihiro  
2-402, Fuchudanchi 28-1, Harumicho, 1-chome  
Fuchu-shi Tokyo(JP)

**(72) Inventor:** Kasahara, Yasushi  
310, 4-4, Nagayama 3-chome  
Tama-shi Tokyo(JP)

**(74) Representative:** Silverman, Warren et al,  
HASLTINE LAKE & CO. Hazlitt House 28 Southampton  
Buildings Chancery Lane  
London WC2A 1AT(GB)

**(54) Method of measuring polynucleotide and reagent kit for use therein.**

**(57)** Disclosed is a method of measuring a polynucleotide such as DNA and RNA in a body fluid which does not require immobilisation of the test sample and whereby the polynucleotide is easily determined, wherein an immobilised single-stranded polynucleotide which is labelled is hybridised with the single-stranded polynucleotide to be measured, the double-stranded polynucleotide formed by hybridisation is broken by means of a restriction enzyme and the labelling substance present is then determined as a measure of the polynucleotide being determined.

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EP U 142 299 A2

1 "METHOD OF MEASURING POLYNUCLEOTIDE AND REAGENT KIT FOR  
FOR USE THEREIN"

This invention relates to a method of measuring polynucleotide, i.e. deoxyribonucleic acid (DNA) or  
5 ribonucleic acid (RNA), in a fluid sample, in particular a body fluid.

The measurement of a DNA in a sample of human serum provides significant results when testing for a viral infection or a hereditary disease. A conventional  
10 method of detecting a particular DNA comprises denaturing of a double-stranded DNA (d-DNA) in a sample under investigation to produce a single-stranded DNA (s-DNA), immobilising the s-DNA on a carrier material, hybridising the immobilised s-DNA with a radioisotope-labelled s-DNA,  
15 removing unreacted radioisotope-labelled s-DNA, and then measuring radioactivity of the immobilised material. Since this method comprises many processes including immobilisation of each sample, it is complicated to carry out and hence the procedure is time consuming.

20 It is an object of this invention to provide a simpler method of measuring a polynucleotide.

According to this invention, there is provided a method of measuring a polynucleotide which is either single stranded as such or a single stranded  
25 polynucleotide obtained from a double stranded polynucleotide to be measured, which comprises, contacting the single-stranded polynucleotide to be measured with an immobilised labelled single-stranded polynucleotide which is able to hybridise with said  
30 single-stranded polynucleotide in aqueous solution to produce a double-stranded polynucleotide, breaking the double-stranded polynucleotide thereby obtained by means of a double-stranded polynucleotide restriction enzyme, and measuring the amount of labelling substance in the  
35 solution obtained and/or on immobilised material, as appropriate as a measure of said single stranded and/or said double stranded polynucleotide to be measured.

1 This invention also provides a reagent kit for use  
in said method comprising the aforesaid immobilised  
labelled single stranded polynucleotide and a restriction  
enzyme.

5 The method of this invention uses as starting  
material a single-stranded polynucleotide, hereinafter  
termed s-polynucleotide to be measured, which may be  
derived from a double stranded polynucleotide in which  
10 case its quantity is a measure of the polynucleotide from  
which it has been obtained. The s-polynucleotide to be  
measured may be s-DNA or s-RNA. When the fundamental  
polynucleotide in a sample to be determined is  
double-stranded, it is necessary that the polynucleotide  
be denatured by alkaline treatment using a sodium  
15 hydroxide solution or heating to convert it to  
single-stranded polynucleotide. Test materials which may  
be subjected to this method include human serum, urine  
and tissue extracts. When a protein is combined with  
the polynucleotide in a sample, such as serum, the  
20 protein is preferably separated off by using, for  
example, a protease.

The immobilised single-stranded polynucleotide  
(labelled s-polynucleotide) incorporates or has bound  
thereto a labelling substance and is able to hybridise  
25 with the s-polynucleotide to be measured. Hence, this  
labelled s-polynucleotide acts as a probe which can  
hybridise with the s-polynucleotide to be measured. The  
s-polynucleotide to be labelled may be produced by  
denaturation of a double-stranded polynucleotide  
30 containing the s-polynucleotide to be measured by means  
of alkaline treatment or heating. It may also be  
produced by a known solid phase polynucleotide synthesis  
method or by a genetic synthesis method accomplished with  
γ-DNA using a plasmid. Some s-polynucleotides have, in  
35 themselves, found commercial use.

The labelling substance which is used may be a  
radioisotope, a fluorescent material, a luminescent

- 1 material, a magnetic material, an enzyme, a prosthetic group of such an enzyme, a coenzyme, or an enzyme inhibitor or activator. Radioisotopes which may be used include  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$  and  $^{125}\text{I}$ . Fluorescent
- 5 materials which may be used include fluorescent reagents, such as rhodamine, fluorescein, methylcoumarin and dansyl chloride. Luminescent materials which may be used include chemically luminescent materials such as isoluminol and luminol, and a biologically luminescent
- 10 material such as luciferine-luciferase. Enzymes, for example glucose oxidase, peroxidase and alkaline phosphates, whose activities can easily be measured are preferably used. Since processes for measuring a polynucleotide include heating, the enzyme is preferably
- 15 thermostable. Should an enzyme which is not thermostable be used, hybridisation may also be carried out at  $35$  to  $40^\circ\text{C}$ . Prosthetic groups include flavine adenine dinucleotide which is an active site of a glucose oxidase, and its amount can be determined by measuring
- 20 the activity of a holoenzyme after reaction with an apoenzyme. Coenzymes which may be used, include NADH,  $\text{NADPH}_2$ , aminopyrophosphate, pyridoxal phosphate, ADP and ATP.

The carrier material of the labelled

- 25 s-polynucleotide may be Sepharose, Sephadex (Registered Trade Marks), cellulose gel, ion-exchange resin, filter paper, nitrocellulose, nylon, polystyrene or polyacrylamide.

Immobilisation may be carried out by following a

- 30 general immobilisation method or a general polynucleotide synthesis route. In such a procedure, an oligonucleotide is first allowed to bind to a carrier material, and then, the s-polynucleotide is allowed to bind to the oligonucleotide by using a 3'-nucleotidase.
- 35 An amino group or carboxyl group is introduced to the s-polynucleotide, and then it is allowed to bind to a carrier material having carboxyl groups or amino groups

1 by using a reagent for peptide synthesis such as  
 dicyclohexyl carbodiimide or carbonyldiimidazole. When  
 both the s-polynucleotide and a carrier material have  
 amino groups, they can be combined by using for example  
 5 cyanuric chloride or glutaraldehyde. When both the  
 materials contain SH groups, they can be combined by  
 using a reductant or a reagent having maleimide group.  
 A physical adsorption method may also be employed for the  
 immobilisation. For example, nitrocellulose is immersed  
 10 in a solution of the s-polynucleotide for a prescribed  
 time and then washed. Finally, the immobilised product  
 may also be produced by connecting nucleotides  
 successively on a carrier material using a solid phase  
 synthesis method.

15 Introduction of the labelling substance into the  
 s-polynucleotide may be carried out by following a  
 conventional method. The introduction is usually  
 carried out prior to the immobilisation. However, when  
 the labelling substance is a large molecule like an  
 20 enzyme, the introduction is preferably carried out after  
 the immobilisation.

The s-polynucleotide to be measured is contacted  
 with the labelled s-polynucleotide in the solution phase.  
 The contacting time is usually about 0.5 to 40 hours.  
 25 The temperature of the solution is preferably from 20 to  
 70°C, and the pH is usually 5 to 9.

The double-stranded polynucleotide restriction  
 enzyme then used (hereinafter abbreviated as "restriction  
 enzyme") is preferably one which is specific for  
 30 double-stranded polynucleotides, and preferably  
 recognises a short polynucleotide chain. Moreover two or  
 more different restriction enzymes may be employed. The  
 restriction enzyme is usually added to the solution after  
 the hybridisation, although it may be added together with  
 35 or prior to the s-polynucleotide to be measured.

After the restriction enzyme reaction, the  
 immobilised material is separated from the solution, if

1 necessary, and the residual labelling substance in the  
 solution or of the immobilised material, as appropriate,  
 is measured. The measurement may be carried out by a  
 known method. For example, radioactivity of this  
 5 material may be determined by using a scintillation  
 counter or a Geiger counter in the case of a radioisotope  
 labelling element.

Characteristically, the method of the invention  
 does not need the immobilisation of the s-polynucleotide  
 10 to be measured, and is easy to carry out. Instead,  
 standard reagents, and optionally devices, can be made  
 available as a kit. A polynucleotide such as DNA can then  
 be easily detected or determined by using this kit.

The following Examples illustrate this invention:

15 EXAMPLE 1

(1) Preparation of HBV-DNA Probe

500 ml of a pool serum from chronic B-type  
 hepatitis patients were centrifuged at 9000 rpm for 15  
 minutes, and the supernatant was further centrifuged  
 20 using an ultracentrifuge at 4°C at 100,000 xg for 5 hours  
 to collect HBV particles as a pellet. This pellet was  
 dissolved in 10 ml of 0.01 M tris-HCl buffer solution of  
 pH 7.5 containing 0.1 M NaCl, 1 mM EDTA, 0.1% by weight  
 2-mercaptoethanol and 0.1% by weight of BSA. 5 ml of the  
 25 virus solution were stored, and the remaining 5 ml were  
 centrifuged at 100,000 xg for 5 hours to obtain a pellet  
 again.

The pellet was treated with 200 µl of 10 mM  
 tris-HCl 0.1 M NaCl pH 7.5 solution containing 0.5% by  
 30 weight NP-40, and DNA polymerase was thereby activated.  
 50 µl of 0.08 M MgCl<sub>2</sub> 0.2 M tris buffer solution of pH  
 7.5 containing 1 mM dATP, 1 mM dTTP, 2.5 µM <sup>32</sup>PdGTP and 2.5  
 µM <sup>32</sup>PdCTP were added to this solution which was allowed  
 to warm up for 3 hours. Then, the solution was layered  
 35 over 30% by weight sucrose solution placed in a  
 centrifuge tube, and centrifuged at 50,000 rpm for 3  
 hours using a SW 65 rotor (made by Beckman Co.) to obtain

1 a pellet. This pellet was treated with a protease, and  
two extractions from the solution were carried each with  
phenol. The extracts were combined and placed on 5 to  
20% by weight sucrose solution gradient, and centrifuged  
5 at 50,000 rpm for 3 hours. 15 S<sup>32</sup>PDNA fractions were  
collected, and pooled. 15 S<sup>32</sup>PDNA was precipitated from  
the pooled fractions by adding ethanol, and dried to  
obtain the target HBV-DNA.

(2) Preparation of DNA Probe by Nick Translation

10 1 µg of the above HBV-DNA was added to 100 µl of  
50 mM tris-HCl buffer solution of pH 7.5 containing 5 mM  
MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 5 µM dTTP, 5 µM dGTP, 5 µM  
-<sup>32</sup>P-dCTP (relative radioactivity 100-700 Ci/mmol) and 5  
µM -<sup>32</sup>P-dATP (relative radioactivity 100-700 Ci/mmol).  
15 100 pg DNase I and 100 pg DNA polymerase I were added to  
the solution which was incubated at 15°C for 90 minutes.  
Extraction of radioactive HBV-D-DNA from the solution was  
carried out by using phenol, and the extract was purified  
by using a Sephadex G-50 column.

20 1 mg of this HBV-d-DNA (1 ml) was mixed with 5 mg  
of single-stranded M13-phage DNA (2 ml). 8 ml of  
formamide were added to the mixture which was then boiled  
for 5 minutes. Subsequently, 2 ml of 0.07 M tris-HCl  
buffer solution containing 2 M NaCl and 15 mM EDTA (pH  
25 7.5) were added to the solution which was then warmed at  
50°C for 4 hours and then at 60°C for 1 hour.

The reaction mixture obtained was separated by  
gel-filtration using Bio-Gel A50 m, and hybridised DNA  
and unreacted DNA were removed. The first peak fraction  
30 near the void fraction was collected, and NaCl powder was  
dissolved therein in a concentration of 0.1 M. 100%  
ethanol in a volume twice that of the solution was added,  
and the solution obtained allowed to stand at -70°C for 2  
hours. Subsequently, the solution was centrifuged at  
35 17,000 xg for 10 minutes, and the precipitates which  
formed were collected. The precipitates were dissolved  
in 50 ml of 0.001% Phenol Red solution containing 0.1 N

1 NaOH and 0.25 mM EDTA, and purified by gel-filtration  
using Bio-Gel A50 to obtain the target HBV-s-DNA.

Meanwhile, 1  $\mu$ g HBV, 100 pg DNase I and 100 pg DNA  
polymerase I were added to 100  $\mu$ l of 50 mM tris-HCl  
5 buffer solution of pH 7.5 containing 5 mM  $MgCl_2$ , 10 mM  
2-mercaptoethanol, 5  $\mu$ MdTTP, 5  $\mu$ MdGTP, 5  $\mu$ MdCTP, 5  
 $\mu$ MdATP, 10  $\mu$ M aminohexyl dATP and 10  $\mu$ M aminohexyl dCTP  
and the solution was incubated at 15°C for 90 minutes.  
Extraction from the solution was carried out by using  
10 phenol, and the extract was purified by using Sephadex  
G-50 column to obtain a modified HBV-d-DNA.

This d-DNA was converted to s-DNA by following the  
aforementioned procedure.

### (3) Preparation of Immobilised Material

15 A polyuracil nucleotide was allowed to bind to  
CNBr-activated Sepharose gel beads. The radioactive  
HBV-s-DNA was added to the gel beads, and the 3'-terminal  
position of the polyuracil was allowed to bind to  
5'-terminal position of the radioactive HBV-DNA by adding  
20 RNA ligase. The gel beads were sufficiently washed with  
50% dimethylformamide containing 0.2 M NaCl to yield the  
required immobilised material.

1.0 ml of d-DNA restriction enzyme solution (Bgl  
II, Ava II, Hae II, Hae III, Hap II, Hinc II - each 1  
25 U/ml, 10 mM tris-HCl, 7 mM  $MgCl_2$ , 70 mM NaCl, 7 mM  
2-mercaptoethanol, pH 7.5) was added to the immobilised  
material, and allowed to react at 37°C for 1 hour.  
Then, the immobilised material was sufficiently washed  
with the same dimethylformamide solution, and HBV-d-DNA  
30 was completely removed.

### (4) Measurement of Sample

100  $\mu$ l of 0.5 N NaOH solution were added to 100  $\mu$ l  
of the serum of a HB viral hepatitis patient and  
stirring at ambient temperature was carried out for 10  
35 minutes. Subsequently, the solution was neutralised by  
adding 100  $\mu$ l of 0.5 N HCl, and 200  $\mu$ l of 200  $\mu$ g/ml  
proteinase K solution were then added. The solution was



1 allowed to react at 70°C for 1 hour. The immobilised materials prepared in step (3) were suspended in 1 ml of the solution. The mixture was allowed to stand overnight at 37°C. The mixture was centrifuged, and the  
5 supernatant was removed. 1.0 ml of d-DNA restriction enzyme solution (Bgl II, Ava II, Hae II, Hae III, Hap II, Hinc II - each 1 U/ml, 10 mM tris-HCl, 7 mM MgCl<sub>2</sub>, 70 mM NaCl, 7 mM 2-mercaptoethanol, pH 7.5) was added, and allowed to react at 37°C for 1 hour. After the  
10 reaction, the resulting mixture was centrifuged, and the radioactivity of 500 µl of the supernatant was measured by using a scintillation counter.

The radioactivity reading was converted to dilution ratio of serum using the previously obtained  
15 standard plot of Fig.1 on which the ordinate axis indicates the counting rate of radioactivity, and the abscissa indicates dilution.

#### EXAMPLE 2

##### (1) Preparation of Immobilised Material

20 A solution containing 500 µg of aminohexyl-induced HBV-s-DNA was dropped onto a nitrocellulose filter (5 x 5 cm) so as to permeate all over the filter. The filter was dried by heating under reduced pressure. Subsequently, the filter was washed sufficiently with 0.2  
25 M NaCl, and immersed in 0.1 M carbonate solution of pH 8.0.

100 mg of N<sup>6</sup>-(6-carboxyhexyl)-adenine flavine dinucleotide were dissolved in 10 ml of dimethylformamide. 50 mg of N-hydroxysuccinimide and 70  
30 mg of water-soluble carbodiimide were added to this solution, and reaction was allowed to take place at ambient temperature for 1 hour. After the reaction, this solution was allowed to permeate all over the filter which was allowed to stand at ambient temperature for 2  
35 days. This filter was washed sufficiently with 0.1 M carbonate solution, and, in this way, unreacted aminohexyl adenine flavine dinucleotide was removed.

## 1 (2) Measurement

100  $\mu$ l of 0.5 N NaOH were added to 100  $\mu$ l of the serum of a HBV viral hepatitis patient, and stirred at ambient temperature of 10 minutes. Subsequently, the solution was neutralised by adding 100  $\mu$ l of 0.5 N HCl, and 200  $\mu$ l of 200  $\mu$ g/ml of proteinase K were then added. The solution was allowed to react at 70°C for 1 hour, and then 200  $\mu$ l of saturated phenol-chloroform solution (1:1) were added. 200  $\mu$ l of the aqueous layer separated off therefrom were dropped on to the above filter (1 x 1 cm), and allowed to react at 60°C for 2 hours. 1.0 ml of d-DNA restriction enzyme solution (Hae III, Hinc II, Xba I, Bgl II, Ham HI, Ava II, Alv I - each 1 U/ml, 20 mM tris-HCl, 7 mM MgCl<sub>2</sub>, 70 mM NaCl, 7 mM 2-mercaptoethanol, pH 7.5) was added, and allowed to react at 37°C for 15 minutes. After the reaction, the filter was removed, and 200  $\mu$ l of a substrate solution (0.025% by weight ABTS, 1.8%  $\beta$ -D-glucose, 7  $\mu$ g/ml POD, 0.1 M phosphate, pH 6.0) containing 10  $\mu$ g of apoglucose oxidase were added. Rate assay was carried out by measuring the increase of absorbance at 420 nm.

The reading obtained was converted to dilution ratio of serum using the previously obtained standard plot of Fig.2 on which the abscissa indicates dilution ratio of serum, and the ordinate indicates absorbance.

1. Claims:

1. A method of measuring a polynucleotide which is either single stranded as such or a single stranded polynucleotide obtained from a double stranded polynucleotide to be measured, which comprises, contacting the single-stranded polynucleotide to be measured with an immobilised labelled single-stranded polynucleotide which is able to hybridise with said single-stranded polynucleotide in aqueous solution to produce a double-stranded polynucleotide, breaking the double-stranded polynucleotide thereby obtained by means of a double-stranded polynucleotide restriction enzyme, and measuring the amount of labelling substance in the solution obtained and/or on immobilised material, as appropriate as a measure of said single stranded and/or said double stranded polynucleotide to be measured.

2. The method of claim 1, wherein said single-stranded polynucleotide to be measured is a single-stranded deoxyribonucleic acid or a single-stranded ribonucleic acid.

3. The method of claim 1 or 2, wherein said labelling substance is selected from radioisotopes, fluorescent materials, luminescent materials, magnetic materials, enzymes, prosthetic groups thereof, coenzymes and enzyme inhibitors.

4. The method of claim 3, wherein the labelling substance is a large molecule substance and has been introduced into the single-stranded polynucleotide prior to immobilisation thereof.

5. The method of any one of claims 1 to 4, wherein the single-stranded polynucleotide which is labelled has been obtained by denaturation of a double stranded polynucleotide containing the single stranded polynucleotide to be measured.

6. The method of any one of claims 1 to 3, wherein the single stranded polynucleotide which is

1 labelled has been obtained by a solid phase  
polynucleotide synthesis method or by genetic synthesis  
with  $\gamma$ -DNA using a plasmid.

7. The method of any one of the preceding claims,  
5 wherein the carrier material is selected from Sepharose,  
Sephadex, cellulose gel, ion exchange resin, filter  
paper, nitrocellulose, nylon, polystyrene and  
polyacrylamide.

8. The method of any one of the preceding claims,  
10 wherein said contacting is carried out at from 20 to 70°C  
at a pH of from 5 to 9 over a period of from 0.5 to 40  
hours.

9. The method of any preceding claim, wherein the  
restriction enzyme is added to the aqueous solution after  
15 hybridisation has been effected therein.

10. A kit of reagents for use in the method of  
any preceding claim which comprises an immobilised  
labelled single stranded polynucleotide which is able to  
hybridise with a single-stranded polynucleotide in an  
20 aqueous solution to produce a double-stranded  
polynucleotide and a restriction enzyme.

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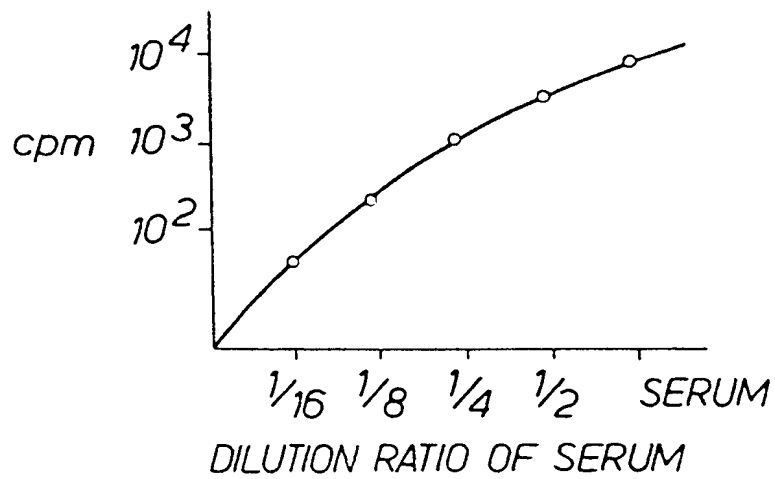


Fig. 1.

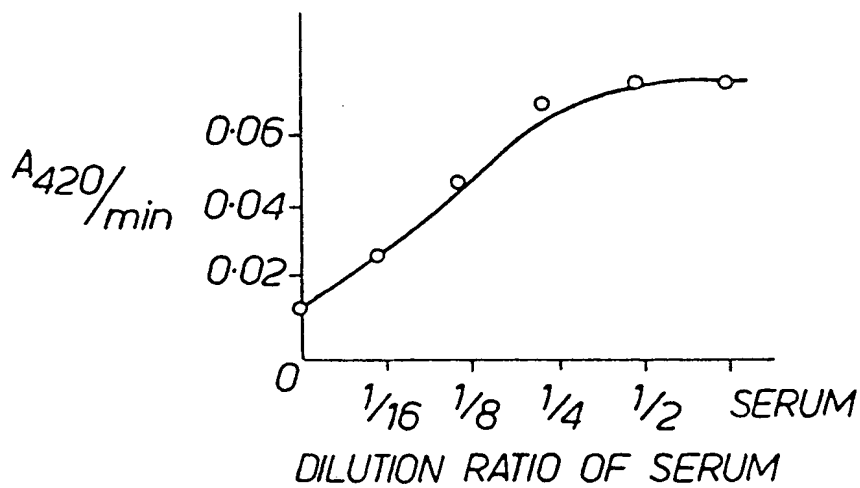


Fig. 2.

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(71)

Applicant: FUJIREBIO KABUSHIKI KAISHA also trading  
as FUJIREBIO INC.  
6-7, Shimoochiai 4-chome Shinjuku-ku  
Tokyo 161(JP)

(72)

Inventor: Ashihara, Yoshihiro  
2-402, Fuchudanchi 28-1, Harumicho, 1-chome  
Fuchu-shi Tokyo(JP)

(72)

Inventor: Kasahara, Yasushi  
310, 4-4, Nagayama 3-chome  
Tama-shi Tokyo(JP)

(74)

Representative: Silverman, Warren et al,  
HASLITINE LAKE & CO. Hazlitt House 28 Southampton  
Buildings Chancery Lane  
London WC2A 1AT(GB)

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Method of measuring polynucleotide and reagent kit for use therein.

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# EUROPEAN SEARCH REPORT

App. No. 84 30 7289

EP 84 30 7289

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	EP-A-0 070 687 (STANDARD OIL CO.) * Page 2, line 23 - page 3, line 18; page 4, lines 4-24; page 6, line 20 - page 7, line 14; page 11, lines 2-30 *	1-3,5,7	C 12 Q 1/68
X	US-A-4 351 901 (C.P. BAHL) * Abstract; column 3, lines 21-36; column 5, lines 4-19; column 13, lines 2-30; column 9, example 1 *	1-3,8	
E	EP-A-0 123 513 (AMERSHAM INTERNATIONAL) * Page 2, line 29 - page 3, line 18; page 12, lines 20-25; page 18, example 2; page 24, claim 1 *	1-3,7-9	
Y	US-A-4 302 204 (G.M. WAHL et al.) * Column 1, line 58 - column 2, line 14; column 2, lines 35-41; column 6, examples I,II; column 9, example 8; column 10, example 10; column 12, lines 18-28 *	1-3,5,7 -9	
Y	WO-A-8 301 459 (ORION-YHTYMÄ OY) * Page 3, lines 12-33; page 8, line 24 - page 9, line 16; page 26, lines 3-24 *	1-3,5,7 -9	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 Q 1/00 C 07 H 21/00 C 12 N 15/00
Y	US-A-4 358 535 (S. FALKOW et al.) * Column 1, line 58 - column 2, line 17; column 2, lines 32-61; column 4, lines 40-47; column 9, lines 26-58; column 10, lines 3-23 *	1-3,5,7 -9	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 25-11-1987	Examiner VAN BOHEMEN C.G.
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